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FRESHWATER AND MARINE ALGAE ACUTE TOXICITY TEST

(a) Purpose. (1) A unicellular alga is used as a model system initially to estimate the concentration of a chemical which could affect survival and growth of freshwater or marine algae. Because generation times for unicellular algal species are measured in hours, this relatively short test can assess effects over several generations. Results allow the assessment of effects on initial organisms from short term exposures and give an indication of the effect on algal populations.

(2) Many different protocols for algal tests are available. This growth test is easy to perform and gives reproducible results with the recommended species. This Test Guideline can be adapted for other algal species. If such an adaptation is used, a description of the method should be provided with the test report.

(b) Definitions. The following definitions apply to this guideline:

(1) "EC-X" means the experimentally derived chemical concentration that is calculated to effect X percent of the test criterion.

(2) "Growth rate" means the rate at which the algal population grows, estimated by the increase in cell numbers or biomass over a specified period of time.

(3) "Inhibition" means any decrease in the growth rate of the population of test algae compared to the controls.

(4) "Limited water-soluble substances" means chemicals which are soluble in water at less than 1,000 mg/l.

(5) "Readily water-soluble substances" means chemicals which are soluble in water at a concentration equal to or greater than 1,000 mg/l.

(c) Test procedures--(1) Summary of the test.

(A) The procedures for the preparation of the algal suspension, the stock solution of the test chemical, and the test media, are dependent on the solubility of the chemical. Modifications in the testing procedure may be necessary due to the chemical's solubility in water. For chemicals with low solubility under test conditions, it may not be possible to quantitatively determine the EC-50.

(B) For purposes of the test, algae are grown in Erlenmeyer flasks in an environmentally controlled growth chamber. The test is started when an algal suspension in nutrient solution (10^4 or $2x10^4$ cells/ml) and the appropriate test chemical dilutions are placed in the flasks. Algal growth is measured at 24-hour intervals for at least 96 hours. A Coulter Counter, cell counting chamber, or other appropriate instruments may be used to

determine population density. The data are used to define the concentration-response curve and the time-growth curve, and to calculate the EC-50 and the EC-0 (no-effect level) for the chemical.

(2) [Reserved]

(3) Range-finding test. A range-finding test should be conducted to establish test chemical concentrations for the definitive test.

(4) Definitive test. (i) The definitive test is used to determine time-growth and concentration-response curves, as well as the EC-50 and EC-0 of the test chemical. The testing method will vary slightly depending upon whether the chemical is readily water-soluble, of limited solubility, and/or volatile.

(ii) Criteria for a valid definitive test. (A) Algae in the control flasks should exhibit log phase growth within 48 hours of test initiation and should produce a population density of at least 10^5 cells/ml in 96 hours.

(B) At 96 hours, one test concentration should show no significant decrease in growth rate and one concentration should show greater than a 50 percent decrease in growth rate relative to the control.

(C) The pH of each test solution should be measured before use and, if necessary, adjusted to 7.5 $\pm$ 0.2 for

freshwater and 8.0 ± 0.1 for marine species using HCl or NaOH.

(D) The concentration of the test chemical in the medium should be determined before and after the test.

(E) No more than 20 percent of the test chemical should be lost by volatilization. If more is lost (or is anticipated to be), the test should be conducted in closed flasks. Carbon dioxide limitations resulting from the closed flasks typically result in population densities lower than the standard in (A) above.

(iii) Test procedures dependent on solubility.--(A) Readily water-soluble chemicals. When readily water-soluble chemicals are tested, the following procedures are recommended:

(1) Just prior to testing, a stock solution of the readily water-soluble chemical should be prepared with micropore-filtered (0.45 um pore size) medium. The concentration of the stock solution should be twice as high as the highest concentration to be used in the test. From this stock solution at least five dilutions should be made. The dilutions should be in a geometric series with a ratio of 10 or n/10 (where n = number of dilutions). The highest concentration should result in at least 50 percent growth inhibition while the lowest concentration should

result in no significant difference ($\alpha = 0.05$) from the growth in the controls.

(2) The algal suspension for testing readily-soluble chemicals should have a density of approximately 2×10^4 cells/ml of nutrient solution. The cell density should be quantified prior to beginning the test.

(3) The test should be carried out in triplicate (i.e. three replicate flasks per concentration). The test begins by transferring 50 ml of algal suspension to 250 ml Erlenmeyer flasks. Then, in sequence of increasing concentrations, a 50 ml volume of each prepared dilution is added to the appropriate flask. The control flasks receive 50 ml of nutrient solution. The flasks are then gently shaken and placed in the growth chambers. The flasks should be shaken by hand at least once every day; this is particularly important for non-motile species. The algal concentration in samples from each flask is determined at intervals of at least 24, 48, 72 and 96 hours after the start of the test and the number of cells or biomass (dry weight per ml) is calculated for each sample.

(4) If a fluorometer or spectrometer is used to calculate cell number or biomass, the measurements

will be inaccurate during the first 24 hours of the test period. Therefore, a calibration curve of meter readings of cell numbers versus test concentrations should be plotted. Calculations should be limited to the straight portion of the calibration curve.

(B) Limited water-soluble chemicals. When chemicals with limited water-solubility are tested, the following modifications of the above procedure are recommended:

(1) Just prior to testing, stock solutions of test chemicals should be prepared with a suitable organic solvent. Each stock solution should be 10^4 times more concentrated than the highest concentration to be tested. The amount of solvent necessary to dissolve the chemical should not exceed 0.1 ml/l of stock solution.

(2) The algal suspension should have a density of approximately 10^4 cells/ml. Otherwise the test should be conducted as described for readily-soluble chemicals.

(3) Each test flask should contain 100 ml of algal suspension and a 10 µl aliquote of the diluted chemical stock. A series of dilutions is made of the chemical stock so that when 10 µl of each dilution is combined with 100 ml of algal suspension the desired test concentrations are obtained. Two controls should be included, one possessing the solvent (10 µl of 0.1 ml/l) and no test chemical, and the second without either.

(iv) Test procedures dependent on volatility. When volatile chemicals are tested, the following modifications of the procedures in Sec(4)(iii)(A) and (B) are recommended. The test is performed as described above (depending on the chemical's solubility in water) except that 250 ml conical flasks with ground glass stoppers should be used. These flasks are filled as described above with the algal suspension and test chemical solution. For each measurement interval the complete contents of the flasks should be used. Therefore, it is necessary to use a sufficient number of flasks to allow for this destructive sampling with time.

(5) Test results. (i) The results of the measurements should be tabulated. Growth curves resulting from the experiment should be drawn on semi-logarithmic paper.

(ii) The reported chemical effects on algae should include both the EC-50 and EC-0. A number of methods have been described for determining the EC50 and EC0. Two examples are provided.

(A) EC-50 and EC-0 determined from the specific maximum growth rate (u). The mean value of the three replicate measurements and the 95 percent confidence limits should be calculated for the 24, 48, 72, and 96 hour sampling times,and plotted as in Figure 1.

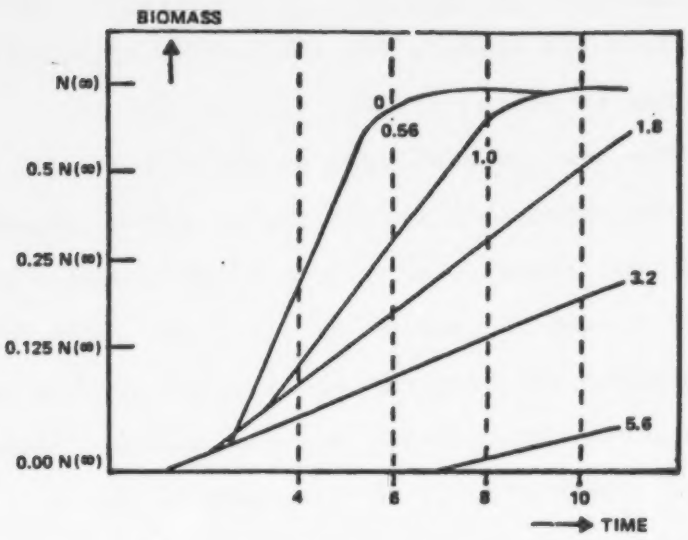

Figure 1. Theoretical example of a result of a toxicity test with algae. The growth curve is plotted on a simple logarithmic scale. The figures give the concentrations of the test compound in weight per volume. The broken lines show the measurements. N(∞) is the maximum cell density that can be achieved.

Two values are selected from the log-linear portion of the curve and the specific growth rate (u) is calculated according to the following formula:

$$u = \frac{\ln (N_2/N_1)}{(t_2 - t_1)}$$

where:

N_1 = the lower cell number chosen in the log phase at t_1

N_2 = the higher cell number chosen in the log phase at t_2

t = time in hours

u = maximum specific growth rate

The percent reduction of the growth rate compared to the control can be calculated:

$$\text{"\% inhibition"} = 1 - (u(tox)/(u(b))) \times 100$$

where:

u(tox) = the growth rate in the presence of the chemical

u(b) = the growth rate in the control

The calculated percentages ("% inhibition") are plotted against the log concentration as shown in Figure 2. The highest concentration showing no difference from the control (a) and the EC50 (b) can then be read from this graph.

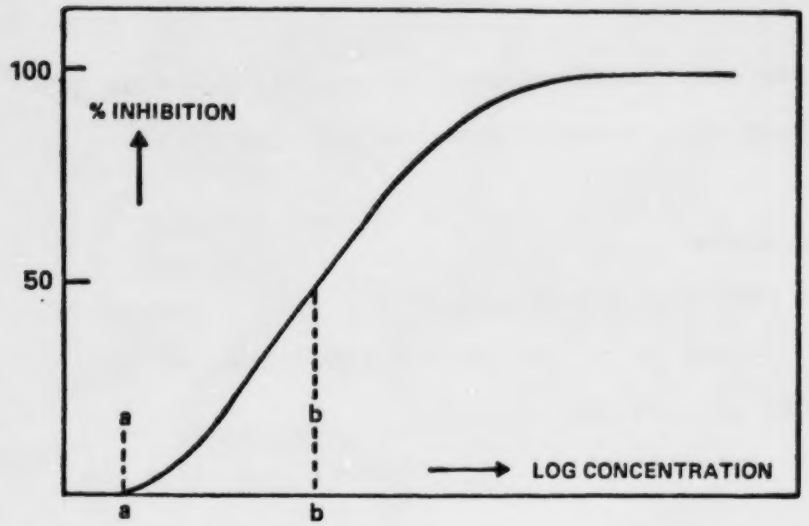

Figure 2. Theoretical example of the relation between the logarithm of the concentration of the chemical and the percentage inhibition.

(B) EC-50 determined from the mean relative growth rate. (1) The mean relative growth rate (RGR) during log phase growth for each culture can be calculated as follows:

$$RGR = (\log_{10} n_2 - \log_{10} n_1)/(t_2 - t_1)$$

where:

n_1 = number of cells/ml at t_1

n_2 = number of cells/ml at t_2

t = time (hours)

(2) The mean relative growth rates (RGR) of the controls and treatment along with the 95 percent confidence intervals (95% CI) are calculated. Treatment RGR values that are greater than the control upper 95 percent confidence interval will be ignored. Those above the mean control RGR but within the 95 percent confidence interval should be assumed to equal the control mean. The treatment RGR values are then expressed as a percentage of the control mean RGR. The percentage values including the highest concentration of test compound at which growth is 100 percent that of control (if values for all higher concentrations are less than 100 percent) and the lowest concentration with a value of 0 percent (if values for higher concentrations are less than or equal to 0 percent) are included in a probit analysis to determine the EC-50 value with its 95 percent confidence limits.

(3) Interpretation of results. Algal populations subjected to chemical stress may quickly establish normal growth rates when the stress condition is removed; consequently, concentrations that produce effects need careful interpretation.

(6) [Reserved]

(d) Test conditions--(1) Test Species--(i) Selection (A) It is recommended that the algae used be fast-growing species that are convenient for culturing and testing. The following species are considered suitable:

Freshwater Algae	Marine Algae
(1) Selenastrum capricornutum.	(1) Skeletonema costatum.
(2) Scenedesmus quadricauda.	(2) Thallassiosira pseudonana.
(3) Chlorella vulgaris.	(3) Isochrysis galbana.

(B) Monocultures of algae are required, and axenic cultures are recommended.

(ii) Stock culture. (A) The stock cultures are algal cultures that are regularly transferred to fresh medium to act as initial test material. Cultures that are not used regularly should be streaked out on sloped agar slants. The tube cultures should be transferred to fresh medium at least once every two months. Stock cultures taken from agar slants should be subcultured in liquid medium once or twice before use.

(B) The stock cultures should be grown in Erlenmeyer flasks containing the appropriate medium (volume about 100 ml). When the algae are grown at 20°C under continuous cool-white flourescent light, a weekly transfer is recommended. An aliquote removed from a mature culture

transferred with a sterile pipette into a flask of fresh medium, for an approximate 100 fold dilution. The growth rate of a species can be determined from the growth curve. If this growth rate is known, it is possible to estimate at what density the culture should be transferred to new medium. This should be done before the culture is out of log growth.

(iii) Selection of test algae. (A) Algae used in a test should be in an exponential growth phase, with a cell density of at least 10^4 cells/ml. If it is not possible to use the stock culture directly for testing (due to lack of or excessive cell growth) it may be necessary to pre-culture the algae prior to use.

(B) To pre-culture the algae, 200 ml of sterile nutrient solution are added to several 500 ml Erlenmeyer flasks. Sufficient algal suspension from stock cultures is added to give an algal concentration of approximately 10^4 cells/ml ($\pm$ 25 percent). The flasks should be continuously illuminated with cool-white fluorescent light, and the temperature maintained at 20°C. Cultures should be shaken by hand at least once every day; this is particularly important for non-motile species. The cell concentration in the culture should be determined daily so that the desired concentration may be obtained for testing.

(C) Other methods of culturing may also be used. Some algae can be grown rapidly in shake cultures. Bubbling with filtered air containing additional carbon dioxide may also accelerate growth. Furthermore, under the culturing conditions described above, the pre-culture for the algal species recommended in this test, should be optimal for inoculation of test flasks after four to five days. If the algal cultures contain deformed or otherwise abnormal cells (e.g. clumped, chlorotic), they should not be used for the test.

(2) Facilities--(i) Apparatus. (A) In order to avoid contamination with bacteria and other algae, all stock culture maintenance operations should be carried out under aseptic conditions. Cultures should be periodically checked for contamination.

(B) Testing and culturing of algae should be done in an environmentally controlled cabinet or chamber capable of maintaining a temperature of 20°C $\pm$ 2°C and continuous illumination of approximately 150 to 200 uE/m^2 sec.

(C) In addition to normal laboratory apparatus and equipment for algal testing, a counting apparatus (e.g. Coulter Counter, cell counting chamber, fluorometer, spectrophotometer, or colorimeter) to determine cell numbers is also necessary.

(D) The following apparatus and equipment are necessary for algal culturing:

(1) Incubators or climate rooms capable of maintaining temperature and light at the recommended intensity.

(2) Filtering apparatus, accompanying membrane filters, (0.45 um) and flasks.

(3) Inoculation needle.

(4) Sterile graduated pipettes.

(5) pH-meter.

(6) Culture tubes (150 x 18 mm) with stainless steel closures.

(7) Erlenmeyer flasks, 250 ml, with ground glass stoppers, and a 3 liter flask.

(ii) Dilution water. Redistilled or deionized water should be used for preparation of stock, chemical, and nutrient solutions.

(3) Test parameters--(i) Carriers. If solvents, are used, they should not themselves be toxic at the concentrations used and should not affect algal growth. In no case should the concentration of an organic solvent exceed 0.1 ml/l in the highest concentration.

(ii) Lighting. Algae should be kept under continuous, uniform illumination of approximately 150 to 200 uE/m^2 sec. The light source should be cool-white fluorescent lights.

(iii) Inoculation For readily water-soluble and volatile chemicals, an algal suspension containing approximately 2 x 10^4 cells/ml should be used. For chemicals with limited water solubility the algal density should be approximately 10^4 cells/ml. After dilution, both initial algal densities should be approximately 10^4 cells/ml.

(iv) Temperature. The temperature for culturing and testing algae should be 20°C $\pm$ 2°C.

(v) Nutrient media. The recommended media for adequate growth of algae are shown in Table 1 and Table 2. All nutrient solutions can be prepared as concentrated stock solutions using the salts shown in Tables 1 and 2 and stored in the dark and cold. These solutions should be sterilized by filtration or by autoclaving. The medium is prepared by adding the correct amount of stock solutions, or the nutrient salts directly, to sterile distilled or deionized water, to give the final concentrations listed. For solid medium, 1.5 to 2 percent agar (wt/vol) should be added to the medium prior to autoclaving. Other media may be necessary if species other than those above are used.

TABLE 1 MEDIUM FOR FRESHWATER ALGAE

Solution 1 (Nutrient salts)	Amount	
$NaNO_3$	0.5	g
NH_4NO_3	0.33	g
$K_2HPO_4.3H_2O$	0.052	g
$CaCl_2.2H_2O$	0.035	g
$Na_2CO_3.10H_2O$	0.054	g
Fe_3-citrate	0.006	g
Citric acid monohydrate	0.006	g
Trace element solution (sol. 2) (see below)	1.0	ml
Redistilled water made up to	1.0	liter
pH	7.7 $\pm$ 0.3	

Solution 2 (Trace Metals)		
H_3BO_3	2.90	g
$MnCl_2.4H_2O$	1.81	g
$ZnCl_2$	0.11	g
$CuSO_4.5H_2O$	0.08	g
$(NH_4)_6MO_7.4H_2O$	0.018	g
Redistilled water made up to	1.0	liter

Table 2 MEDIUM FOR MARINE ALGAE

Solution 1 (Nutrient salts)	mmol/l	g/kg solution
NaCl	362.661	20.758
Na_2SO_4	24.993	3.477
KCl	8.038	0.587
KBr	0.7249	0.0845
H_3BO_3	0.3715	0.0225
NaF	0.0657	0.0027
$MgCl_2.6H_2O$	47.176	9.395
$CaCl_2.2H_2O$	9.139	1.316
$SrCl_2.6H_2O$	0.0082	0.0214
Solution 2		
$NaHCO_3$*	2.066	0.170
Solution 3 (Trace Metals)***	g/l	umol/l**
$NaNO_3$	4.667	549.09
$Na_2SiO_2.9H_2O$****	3.000	105.60
Na_2 glycerol PO_4	0.667	21.79
Na_2EDTA+	0.553	14.86
H_3BO_3	0.380	61.46
$Fe(NH_4)_2(SO_4)_2.6H_2O$	0.234	5.97
$FeCl_3.6H_2O$	0.016	0.592
$MnSO_4.4H_2O$	0.054	2.42
$ZnSO_4.7H_2O$	0.0073	0.254
$CoSO_4.7H_2O$	0.0016	0.0569
Solution 4 (Vitamins++)		
Thiamine HCl	0.1	0.297
Vitamin B_{12}	0.002	0.00147
Biotin	0.001	0.00409

* Dry, autoclave the $NaHCO_3$ then add to autoclaved solution 1.
** Final concentration in solution
*** Should be autoclaved (50 minutes)
**** Should be neutralized before adding to other solutions. To neutralize, 100 ml of $Na_2SiO_2.9H_2O$(3.0 g/l) is added to 20 ml 1 N HCl and then added to solution.
\+ Na_2 EDTA, $FeCl_2.6H_2O$, $Fe(NH_4)_2(SO_4)_2.6H_2O$ has to be in solution before remaining salts (Mn, Zn, Co) are added.
++ Filter, sterilize, store frozen

(e) Reporting. In addition to a description of the type of test and method, the report submitted to EPA should include the following information.

(1) For the chemical tested: manufacturer, empirical formula, batch number and its degree of purity, chemical characterization, and physical properties.

(2) For the test organisms: origin of innoculum, laboratory culture and strain number, and method of cultivation (including whether and how cultures were aerated and/or shaken).

(3) For the test conditions: date of beginning and end of the test and its duration, temperature, light intensity and light quality in the growth chamber, type of test flask (and if closed), initial pH of test solution, what carrier was used and how much, concentrations of test chemical, and the counting method.

(4) For the results: a tabulation of cell number or biomass (dry weight per ml) for each flask at each sampling period; the plotted time-growth curves for each concentration and the concentration-effect curve; the EC-50 and EC-0 values and the statistical methods used to calculate them; and other observed effects, e.g. stimulation

of growth, algicidal vs. algistatic effects, clumping or chlorosis of cells.

(5) For the laboratory performing the test: the name of the person responsible for carrying out the test (e.g., study director) as well as the name of the person carrying out the test, the name and address of the testing laboratory, and the date and signature of the person responsible for the test.

LEMNA ACUTE TOXICITY TEST

(a) Purpose. This guideline prescribes test procedures and conditions using the freshwater aquatic plant Lemna gibba G3 to develop data on the phytotoxicity of chemicals. The United States Environmental Protection Agency (USEPA) will use data from these tests in assessing the hazard of a chemical to the environment under the Toxic Substances Control Act (TSCA) (P.L. 94-469, 90 Stat. 2003, 15 U.S.C. 2601 et seq.).

(b) Definitions. The definitions in Section 3 of TSCA and Part 793--Environmental Effects Testing Good Laboratory Practice Standards are applicable to this guideline. The following definitions also apply:

(1) "Axenic" means a culture of Lemna fronds free from other organisms.

(2) "Colony" means an aggregate of mother and daughter fronds attached to each other.

(3) "EC-X" means the experimentally derived chemical concentration that is calculated to effect X percent of the test criterion.

(4) "Frond" means a single Lemna "leaf-like" structure.

(5) "Frond mortality" means dead fronds which may be identified by a total discoloration (yellow, white, black or clear) of the entire frond.

(6) "Static-replacement test" means a test method in which the test solution is periodically replaced at specific intervals during the test.

(c) Test procedures -- (1) Summary of the test. (i) In preparation for the test, containers are filled with appropriate volumes of nutrient medium and/or the test solutions. The test is started by introducing Lemna fronds into each of the containers. Nutrient medium and test solutions should be replaced on days 3 and 6 or as needed to prevent nutrient limitation or depletion of the test chemical.

(ii) Colonies should be inspected for changes in frond number and appearance at the beginning of the exposure period (day 0), on days 3 and 6, and at the end of the exposure period (day 7). On day 7, the total number of living and/or dead fronds are counted. Any frond which is visible as a bud when viewed under a hand lens or dissecting microscope should be counted. Concentration response curves are plotted for total frond number, growth rate (as number of fronds per day) and mortality (percentage of dead fronds to total number of fronds). EC-10's, EC-50's, and EC-90's are determined from the curves.

(2) [Reserved]

(3) Range-finding test. (i) A range-finding test should be conducted to establish (A) if definitive testing is necessary and (B) test solution concentrations for the definitive test.

(ii) The recommended procedure is to expose Lemna to a chemical concentration series (e.g., 0.1, 1.0, 10, 100 and 1000 mg/l) plus controls. Three, four-frond colonies should be selected and exposed to equal volumes of each chemical concentration for a period of seven days. The exposure period may be shortened if data suitable for the purpose of the range-finding test are obtained.

(iii) The lowest chemical concentration in a test series, exclusive of controls, should be the lowest concentration which can be analytically quantified. The highest concentration should be at least 1000 mg/l. Replicates are not needed and nominal concentrations of the chemical are acceptable for range-finding. If the calculated EC50 is greater than 1000 mg/l or is less than the analytical detection limit, definitive testing is not necessary. However, replicates and measured concentrations of the appropriate dose are needed to substantiate this result.

(4) Definitive test. (i) The purpose of the definitive test is to determine the EC-10's, EC-50's, and EC-90's for Lemna growth based on total frond number, growth rate and/or frond mortality with a minimum amount of testing beyond the range-finding test.

(ii) At least five concentrations of chemical, exclusive of controls, should be used in the definitive test. The concen-

tration range should be selected to define the concentration response curve between the EC-10 and EC-90. For each concentration and control at least seven replicate containers should be used, each containing 150 ml of test solution and three randomly selected four-frond colonies. Fewer replicates, each containing a greater number of colonies, may be used; however, test containers and solution volumes will have to be adjusted accordingly. The range of chemical concentrations tested should result in the highest concentration affecting at least 90 percent of the fronds and the lowest concentration affecting no more than 10 percent of the fronds compared with the controls.

(iii) Every test should include controls consisting of the same nutrient medium, number of fronds, environmental conditions, and procedures as the test containers except that none of the chemical is added. If a solvent or carrier is used to dissolve or suspend the test chemical, additional controls containing the solvent or carrier should also be included in the test to determine any effect of the solvent or carrier on the plants.

(iv) The colonies should be transferred to freshly prepared test solutions on days 3 and 6. No more than 20 percent of the test substance should be lost by volatilization between replacements. The colonies may have to be transferred more frequently for highly volatile test substances in order to maintain 80 percent of the initial test substance concentration.

Transfer should be done in a clean, draft-free area as quickly as possible to minimize contamination of the colonies.

(v) Observations of frond numbers and appearance should be made of the colonies on day 0, 3, 6, and 7. A dissecting microscope will facilitate observations.

(vi) Concentration response curves should be plotted. These curves can provide the basis for determining the EC-10's, EC-50's, and EC-90's for total frond number, growth rate, and mortality.

(vii) Any change in frond development or appearance such as increase in number (a frond is counted regardless of size as long as it is visible adjacent to the parent frond), necrosis, chlorosis, etc., should be reported. Chlorophyll content of the colonies may be used as an indicator of chlorosis. Any additional observations such as sedimentation of test solution, sinking of fronds, or other abnormalities should also be recorded.

(viii) A randomized complete block design is recommended for the definitive test with blocks delineated within the test chamber. If, for any reason, blocking is not feasible, total randomization within chambers is suggested.

(5) [Reserved]

(6) Analytical measurements -- (i) Chemical. Stock solutions should be prepared just prior to use and diluted with

glass distilled or deionized water to obtain the test solutions. Standard analytical methods, if available, should be used to establish concentrations of these solutions and should be validated before beginning the test. An analytical method is not acceptable if likely degradation products of the chemical, such as hydrolysis and oxidation products, give positive or negative interference. The pH of the test solutions should also be measured prior to and after use.

(ii) Numerical. The number of fronds is counted at the end of the definitive test. Means and standard deviations are calculated and plotted for each treatment and control. Appropriate statistical analyses are used to provide a goodness-of-fit determination for the concentration response curves.

(d) Test conditions -- (1) Test Species. The test species to be used in these tests is Lemna gibba G3. Axenic cultures may be obtained from laboratory cultures or commercial sources. A stock culture grown from a single isolated frond should be used to inoculate all the flasks used in a given test.

(2) Acclimation. Axenic stock cultures should be grown in the aquaria for two weeks (with necessary transfers) prior to being used in a test. Four-frond plants used in a test should be randomly selected from the culturing tank. Inocula should be taken from cultures which are less than two weeks old.

(3) Facilities -- (i) Apparatus. (A) A controlled environment growth chamber or an enclosed area capable of maintaining the specified test parameters (see Section 4 below) is needed.

(B) Laboratory facilities for the mixing and diluting of nutrient medium and a source of distilled or deionized water are needed. An autoclave (for sterilizing glassware and media) and a sterile transfer hood (for maintenance of an axenic Lemna culture) are also necessary. Disposal facilities should be adequate to accommodate spent test solutions and plant materials as well as any bench covering, lab clothing, or other contaminated materials.

(ii) Containers and support media. Test containers may be glass beakers or Erlenmeyer flasks, large enough to hold 150 ml of test solution and the Lemna colonies without crowding for the duration of the test; 250 ml beakers are recommended. All containers should be of the same type and size. If fewer replicates are used, larger containers may be necessary to hold the additional colonies and test solution volume. The ratio of test solution to container volume should not exceed 3:5. For each test concentration and control the same number of replicates should be used.

(iii) Cleaning and sterilization. All glassware and equipment should be cleaned following good laboratory practice. The Nytex screen or inoculating loops used for transferring the Lemna should be disposed of after use or thoroughly cleaned and sterilized before reuse.

(iv) Nutrient media. Hoagland's nutrient medium is recommended for maintaining Lemna cultures and for use as the diluent in the preparation of various concentrations of test solutions. Deionized or distilled water should be used to make the nutrient medium. The medium should be freshly prepared prior to each transfer of Lemna cultures and for the preparation of new test solutions during the course of a test. The pH of the medium should be adjusted to between 4.8 to 5.2 by the addition of 0.1N NaOH for culture maintenance prior to addition of the test chemical. Nutrient media should not contain EDTA, other chelating agents, or organic metabolites such as sucrose.

(v) Carriers. Stock solutions of substances of low aqueous solubility may be prepared by use of organic solvents, emulsifiers or dispersants of low phytotoxicity to plants. When a solvent or carrier is used, a second set of controls should contain the same concentration of the solvent or carrier as that used in the highest concentration of the test substance. The concentration of the solvent or carrier should not exceed 0.1 ml/l.

(4) Test parameters. Environmental conditions should be maintained as specified below:

(i) Temperature at 25 ± 2°C.

(ii) The pH of the nutrient medium between 4.8 and 5.2. Test solution pH may vary from the nutrient medium after the addition of the test chemical and/or carrier (if used). Any such changes should be recorded but not adjusted.

(iii) Continuous irradiation of 400 ± 50 uEinsteins/m^2 sec measured at the test solution or nutrient medium surface.

(e) Reporting. Reporting requirements of part 793-Environment Effects Testing Good Laboratory Practice Standards apply to this guideline. The following data should also be reported:

(i) Source of Lemna and taxonomic verification.

(ii) Description of test chambers, type of lights, size of beakers or flasks used, number of concentrations and replicates per concentration, number of colonies per replicate, solution volumes, physical parameters of growth chambers (e.g., humidity, temperature, and light intensity).

(iii) The ph and concentration of the test chemical in the test solutions prior to use and discarding on day 3, 6 and 7.

(iv) Number of fronds per test concentration and control at the end of the test, the percent inhibition and/or stimulation of

growth rate, and percent frond mortality for each test concentration compared to controls.

(v) If the range-finding test showed that the highest concentration of the chemical tested (not less than 1000 mg/l) had no effect on Lemna, report the results and measured concentrations and a statement that the chemical is not phytotoxic at concentrations less than 1,000 mg/l.

(vi) If the range-finding test showed greater than a 50 percent effect with a test concentration below the analytical detection limit, report the results and a statement that the chemical is phytotoxic below the analytical detection limit.

(vii) Means and standard deviations for frond number, growth rate, and percent frond mortality in each test concentration. In addition, concentration response curve(s) with 95 percent confidence limits delineated, goodness-of-fit determination, and EC-10's, EC-50's and EC-90's identified.

(viii) Methods and data records from chemical and numerical analyses including validation methods and quality assurance procedures.

ES-15
October, 1983

TECHNICAL SUPPORT DOCUMENT

FOR

LEMNA ACUTE TOXICITY TEST GUIDELINE

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Office of Toxic Substances

ES-15
October, 1983

TECHNICAL SUPPORT DOCUMENT FOR LEMNA ACUTE TOXICITY TEST

I. PURPOSE

The purpose of this document is to provide the scientific background and rationale used in the development of Test Guideline EG-23 which uses Lemna to evaluate the phytotoxicity of chemical substances. The document provides an account of the scientific evidence and an explanation of the logic used in the selection of the test methodology, procedures and conditions prescribed in this test guideline. Technical issues and practical considerations relevant to the test guideline are also discussed.

II. Scientific Aspects

A Test Procedures

1. General. Lemna is an excellent organism to use for the detection of phytotoxic effects of chemicals. It is widely distributed in nature and is part of the food chain of waterfowl, fish, and other animals such as muskrats. It has even been proposed as a fodder supplement for cows (Hillman and Culley 1978). Because of this significant role in food chains as well as the growth of duckweed on stagnant and often polluted water, Lemna has gained prominence as a test organism in bioassays (Culley and Epps 1973, Harvey and Fox 1973).

The exposure of plants is often the inadvertent result of leaching of chemicals used for treating objects which are near or in the water, spraying of chemicals on crops or forests, or spilling or other direct or indirect runoff of chemicals into waterways or on soils where they may find their way into the water table. The intentional or unintentional exposure of aquatic plants to potentially

toxic chemicals is important in assessing the quality of water for subsequent plant growth and further uses of the water or plant.

The parameters of growth and mortality were selected to measure the phytotoxicity of chemicals to Lemna for the following reasons:

- Lemna is easily grown under controlled laboratory conditions. Ashby et al. (1928, 1935) first established conditions for the laboratory growth of Lemna and recommended its use as a common laboratory organism for bioassay work. Extensive work since then has established the optimum laboratory conditions for Lemna growth (Czopek 1959, Gorham 1950, Krzechowska et al. 1975, Stanley and Madewell 1976b, Steinberg 1946).
- The use of Lemna in bioassays is well established. Bioassay's have been developed for both flow-through systems (Walbridge 1977, Bishop and Perry 1980) and static systems (OECD 1980, Fromm 1951, 1960, Posner et al. 1977, Watanabe and Takimonto 1977). Fromm (1951, 1960) developed a Lemna bioassay for herbicides based on changes in frond number to replace field testing. Liu and Cedeno-Maldonado (1974, 1979) have done extensive work on developing a bioassay using Lemna to detect residual activity of aquatic herbicides.
- Lemna is sensitive to a number of organic chemicals. A comparison of six common bioassays (chlorophyll inhibition in cucumber cotyledon, algal reproduction inhibition, Lemna minor growth inhibition, oat shoot bioassay, oat root bioassay

and inhibition of cucumber root growth) showed that for the majority of herbicides tested, Lemna was as sensitive as the other bioassays at the same concentration of test chemical (De Cleire et al. 1975). Of the chemicals tested Lemna was most responsive to ammonium ions, phenols, amides, and benzonitriles. Lemna has also been used for chemical testing in the field (Hutchinson and Czyrska 1972, Van Busschbach and Elings 1967, Van der Weij et al. 1971). Bailey (1970) found Lemna to be more sensitive to diuron than oats or Italian ryegrass, with Lemna sensitive to less than 0.12 ppm while the other two plants were unaffected at this level. In addition, Lemna required only 4 days to test compared to 4 weeks for the other two plants. Audia and Preston (1965) found Lemna to be more sensitive than several aquatic plants such as Saggitaria and as sensitive as Azolla and Salvinia when exposed to a variety of algicides. A comparison of Lemna and Azolla sensitivity to paraquat and diquat, showed Lemna to be almost twice as susceptible to both herbicides at concentrations less than 0.01 ppm. At greater concentrations the difference in response between the plants decreased. Lemna also was found to be sensitive to herbicides specific for submergent and floating plants such as Ceratophyllum and Chara, but to be unaffected by herbicides such as dalapon, which are used to control emergent aquatic plants (Newbold 1975). Futhermore, Lemna was even found to be a more sensitive genus to herbicides such as atrazine, diuron, and terbutryne than was Spirodela or

the rooted aquatic plants Azolla and Salvinia (Van der Weij et al. 1971).

- o Lemna may be used to quantify several phytotoxic responses. Not all chemicals which are phytotoxic act upon the same physiological mechanisms in a specific plant. Some chemicals such as monuron, diquat and paraquat inhibit the Hill reaction of photosynthesis or other photosynthetic activities (Parker 1965). Other chemicals such as O-phenanthroline act as metal chelators (Oota and Tsudzuki 1971) or 2,4-dichlorophenoxyacetic acid cause chlorosis and a reduction in cell starch content (Blackman and Robertson-Cuninghame 1955). With chemicals causing such diverse reactions at the cell level no single physiological screening method of determining phytotoxic effects is sufficient. Because the Lemna bioassay is a whole plant test that evaluates the effects of the test chemical on growth, vegetative reproduction, morphology and death it is not so limited. In addition, physiological changes such as chlorophyll content or starch accumulation and/or bioaccumulation of the test chemical or its metabolites also may be evaluated.

The procedures given in the test guideline are in part modified and elaborated from the protocols developed by OECD (1980) and USEPA (1979, 1980) and tested in the laboratory by USEPA (1981). The static-replacement method outlined in this test guideline constitutes the recommended approach. Some previous tests were conducted with a flow-through apparatus rather than as a static or static-replacement test (Walbridge 1977, Bishop and Perry 1980).

However, on the basis of a comparison of five chemicals tested simultaneously with a flow-through and a three-day static-replacement test, the static-replacement test was found to be as sensitive as the flow-through test for the chemicals tested (USEPA 1981). The added advantage of the static-replacement test is that it is simple, inexpensive, and requires less test chemical and nutrient solution to run.

2. Range-Finding Test. The range-finding test is conducted to determine the upper and lower limits of test substance concentrations to be used in the definitive test. It may also indicate that definitive testing is not necessary. In order to reduce the time and cost of the range-finding test, nominal concentrations of the test chemical may be used, no replicates are required and the test duration may be shortened.

The lowest concentration used in the range-finding test should be such that no inhibition or stimulation of growth is evident and chlorosis of fronds is not apparent; while the highest concentration should be such that greater than 90 percent effects are evident. The lowest concentration should be at the lowest limit of analytical detection while the highest concentration used in this test should be at the saturation level (at least 1000 mg/l). If the results of the range-finding test indicate that the chemical is relatively non-toxic (EC-50 is more than 1000 mg/l) or extremely toxic (EC-50 is below the limit of analytical detection), the definitive test would provide no additional data and, therefore, considered unnecessary. However, the testing conditions must be verified as well as the actual test chemical concentrations measured.

3. Definitive Test. The purpose of the definitive test is to determine the EC-10's, EC-50's, EC-90's for frond number, growth rate, and mortality, with a minimum of testing beyond the range-finding test. The concentration range selected is typically based on the results of the range-finding test. At least five concentrations are needed to properly describe the dose-response relationship. The slope and shape of the dose-response curve can give an indication of the mode of action of the chemical and allow estimation of the effects of a given exposure concentration on Lemna.

The primary observations - total number of dead and alive fronds, growth rate, frond mortality and measured chemical concentrations - are needed to accurately plot the dose-response curves from which the EC-10's, EC-50's, and EC-90's are determined.

As discussed by Hammer and Urquhart (1979), it is essential that the investigator randomly assign colonies to flasks and flasks to treatments to assure that each colony has the same chance of receiving any of the treatments (exposure level of test chemical). If more than one chamber is used for a test, replication should occur over chambers (of the same type) to minimize between chamber variance (Hammer and Urquhart 1979). This is recommended because differences in plant response between chambers may be greater than differences resulting from exposure conditions within a chamber.

The test duration should be seven days. Not all chemicals which may be phytotoxic will cause frond chlorosis or necrosis within a shorter period of time. Some test substances may act slowly and affect photosynthetic metabolism or other metabolic processes which would not be

immediately apparent as visible frond damage but only be evident by a reduced or stimulated growth rate. Any such effects should be evident within two generations or seven days which is the length of the test.

4. Analytical Measurements Nominal concentrations are not adequate for the purposes of the definitive test. The chemical concentrations used in the definitive test must be determined with the best practical analytical precision. Stock solutions and test solutions should be prepared just prior to use to minimize problems with storage (e.g., formation of degradation products, adsorption, transformation, etc.). If definitive testing is not required because the chemical elicits an insufficient response at the 1000 mg/l level in the range-finding test, the concentration of chemical in the test solution must be determined to confirm the actual exposure level.

The pH of the test solution should be measured prior to testing and after use to determine if it lies outside of Lemna's optimal range. Although Lemna will grow over a wide range of hydrogen-ion concentrations (McLay 1976), the pH of the nutrient medium is initially adjusted to 4.8-5.2 to prevent nutrient precipitation. However, the pH is not adjusted after the addition of the test chemical to the nutrient medium for the following reasons: the use of acid or base to adjust pH may chemically alter the test chemical, making it more or less toxic; the amount of acid or base needed to adjust the pH may vary from one test solution concentration to the next; and the effect the test substance has on pH may indirectly affect the growth and development of the fronds.

To reduce subjectivity or time required for determining frond size, all fronds, regardless of size are counted as

whole fronds if the frond or bud is evident at the side of the parent frond. Variability in exposure time and subsequent counting or mortality data may be reduced by examining and counting containers sequentially (by concentration) in the concentration order that the fronds were inoculated. Due to the rapid growth of this plant, frond growth should be measured at approximately the same time of day. Concentration-response curves are commonly used for expressing data for bioassays. In this test guideline, the response curves should be expressed in three ways: (1) the direct enumeration of total fronds for a given chemical concentration; (2) the growth rate (K) of fronds; and (3) frond mortality, where the number of dead fronds for a chemical concentration is plotted. All three parameters should be plotted against chemical concentrations. Statistical analysis (goodness-of-fit determination) facilitates accurate EC-10, EC-50 and EC-90 determinations as well as confidence limits for each of the concentration-response curves. The growth rate (K) may be determined by any valid method; the recommended equation is:

$$K = (\log_{10} \text{ fronds on day } d - \log_{10} \text{ fronds on day } 0)/d$$

where $\underline{d}$ is the number of days of the test. The frond number is both living and dead fronds. These numerical analyses provide the quantitation necessary for the Agency to make an evaluation of the phytotoxic potential of the chemical being tested.

B. Test Conditions

1. Test Species. Lemna gibba G3 is the recommended strain to be used in this test guideline as it is more taxonomically stable than many of the other Lemna strains. There is only one L. gibba strain commonly used, strain G3, while L. minor has several strains which have been isolated from the field (Hillman 1961a). Consequently although L. minor may be more commonly used in laboratory studies and may be obtained from natural field populations, it should not to be used in this phytotoxicity test. L. gibba G3 should be obtained as an axenic culture from a reliable source, such as a research laboratory or commercial supply house, who will certify the identity of the plant. Lemna gibba G3 is also the recommended strain for testing under FIFRA (USEPA,1980), OECD (1978), and by UCCES (1979). Lemna has many distinct characteristics which make it particularly useful as a laboratory test organism:

- it will grow easily on inorganic, artificial medium;
- the growth rate of the fronds is such that a new generation is produced every 2-3 days under optimal growth conditions;
- it does not root in a substrate and does not require a solid surface on which to grow as do many other aquatic plants such as Myriophyllum;

- it may be viewed without the use of a microscope, and damage to fronds as well as new frond numbers may be easily assessed (a dissecting microscope or hand lens may facilitate visual estimates of damage and budding);

- it grows over a wide range of light intensities, temperatures, pH's, and a variety of mediums;

- it is easily isolated as a pure culture and grown axenically;

- it may be grown vegetatively from a single frond, thereby reducing genetic variability within a test.

3. Facilities

a. General A growth chamber(s) or adequate space in an environmentally controlled room is needed to maintain a continuous, constant light intensity and temperature. A greenhouse is not considered adequate as fluctuations in environmental conditions may occur and a continuous, constant light intensity and constant temperature are impossible to maintain. Other facilities are needed for cleaning and sterilizing equipment, maintaining stock cultures, preparing solutions and analyzing results, and disposing of test solutions. Equipment such as standard glassware, and analytical apparatus are also needed. Without these facilities the test cannot be adequately performed or evaluated.

b. Test Containers. Erlenmeyer flasks are recommended as test and culture containers. Any flask volume may be used between 250-500 ml. However, for any given test the flasks should be of the same volume so that differences in fluid surface area do not impact on growth or exposure. Flasks should be sterilized and stoppered with sterile plugs (such as cotton stoppers) prior to use. This will minimize possible bacterial contamination yet allow air exchange.

c. Cleaning and Sterilization. Standard good laboratory practices are recommended to remove dust, dirt, other debris, and organic and inorganic residues from the test containers which might confound test results. Prior to use or reuse, all containers, and other glassware or equipment should be washed and, sterilized to minimize contamination.

When fronds are transferred to fresh medium to start the test, they should be rinsed at least twice in distilled or deionized water to prevent algal contamination. If algal contamination of the stock culture becomes severe, a new culture may be grown from the original axenic source culture; or a new axenic stock culture may be sterilized and grown according to the methods of Hillman (1961a).

d. Nutrient Medium. The nutrient medium to be used for the stock cultures as well as the diluent for the test substances is Hoaglands M-medium without EDTA or surcrose. This medium is commonly used for the culture of Lemna species and has given satisfactory results for Lemna gibba G3 growth (UCCES 1980, OECD 1980, Hillman 1961b, Krzechowska et al. 1975). Sucrose and EDTA are omitted from the medium because they have been implicated in the

promotion of flowering in Lemna gibba G3 under long-day conditions (Hillman 1961b). Flowering decreases frond growth (Hillman 1961b) and since flowering of most Lemna species is rare in nature and reproduction is usually vegetative, flowering is to be avoided. EDTA, acting as a metal chelator, may also interact with some test chemicals and alter their availability or toxicity.
Since water hardness has been shown to affect the toxicity of certain chemicals (McKee and Wolff 1963, Parker 1966), only deionized, distilled water should be used to make up the nutrient medium.

4. Environmental Conditions. The temperature specified in the test standard is 25° ± 2°C. Although Lemna is a thermal tolerant organism (Stanley and Madewell 1976b), the optimum temperature for growth lies between 20° - 30°C (Hillman 1961a). Most assay work on Lemna, whether L. gibba G3 or L. minor, has used 25°C as the assay temperature (Hillman 1961b, OECD 1980, USEPA 1980). This temperature produces good growth and a doubling rate of frond number in 2-3 days. Severe fluctuations in temperature may affect Lemna growth and/or subsequent chemical uptake or metabolism. Temperature should be recorded at least hourly to ensure that it does not exceed the specified limits. Inexpensive growth chambers are available which are equipped with automatic recorders at minimal cost. The test guideline specifies a pH of 4.8-5.2 for the nutrient medium, which is the optimum pH that may be obtained using Hoagland's M-medium (Hillman 1961b). The pH is adjusted after autoclaving using 0.1N NaOH. This pH range has also been acceptable for L. gibba G3 growth by several laboratories (Walbridge 1977, USEPA 1980, OECD 1980, UCCES

1980) although it has been grown on pH's as high as 8.0 (Fromm 1951). Frond growth may be affected by variations in pH between test chemical concentrations. The pH is measured as an indication of effects of test chemical additions and as an aid to help evaluate subsequent frond growth. Lemna has been used in testing over a wide range of light intensities. The light intensity specified in this test standard is 400 $\pm$ 50 uEinsteins m^{-2} sec. This intensity is approximately in the middle of the optimum range of light intensities for Lemna growth (USEPA 1980). Continuous light is recommended to accelerate growth. Landolt (1957) found Lemna growth was not inhibited by continuous lighting for long periods of time. Warm, white fluorescent lighting is recommended and should provide irradiation in the range of 400-700 nm to ensure adequate Lemna growth. Light variations will affect Lemna growth. As such it is necessary to maintain uniform and constant radiation. Light intensity should be measured at the surface of the solutions to ensure that all containers are receiving equal light.

III. REPORTING. The sponsor should submit to the Agency all data developed during the test that are suggestive or predictive of phytotoxicity. If testing specifications are followed, the sponsors should report that specified procedures were followed and present the results. If alternative procedures were used instead of those recommended in the test guideline, then the protocol used should be fully described and justified. Test temperature, chemical concentrations, test data, concentration-response curves and statistical analyses should all be reported. The justification for this body of information is contained in this support document.

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